Original Article
A novel dominant-negative mutation of the CSF1R gene causes adult-onset leukoencephalopathy with axonal spheroids and pigmented glia

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Abstract: Adult-onset leukoencephalopathy with axonal spheroids and pigmented glia (ALSP) is a rare autosomal dominant disorder that is caused by mutations in the colony-stimulating factor 1 receptor (*CSF1R*) gene. Functional haplo-insufficiency of the *CSF1R* gene has been considered for the underlying genetic mechanisms. A novel mutation of *CSF1R* and its effects on CSF1R expression or clinical characteristics were explored in an ALSP family. Clinical data and imaging data were collected from the family members with ALSP. Peripheral blood samples were collected for DNA and RNA extraction. Whole-exome sequencing and quantitative PCR were used to identify mutations and to determine the expression of CSF1R. The family had a history of a dominant hereditary pattern. Patients in this family presented motor symptoms, emotional abnormality, or memory impairment at onset. MRI findings showed high hyperintensity signals of T2-weighted imaging in the white matter and atrophy of the corpus callosum. *NOTCH3* gene sequencing ruled out the diagnosis of CADASIL. Whole-exome sequencing identified a novel splice-site mutation (c.2319+1C>A) in intron 16 of the *CSF1R* gene. CSF1R mRNA was significantly decreased (~15%) in the peripheral blood samples of affected patients, which was much lower than the expected 50%. Our findings not only supported the pathological implication of this splice-site mutation but also demonstrated for the first time a dominant-negative effect on CSF1R expression. This report extends the genetic spectrum of ALSP with *CSF1R* mutations and provides evidence for the clinical heterogeneity of ALSP.

Keywords: Colony-stimulating factor 1 receptor, adult-onset leukoencephalopathy with axonal spheroids and pigmented glia, leukoencephalopathy, hereditary diffuse leukoencephalopathy with spheroids, mutation

Introduction

Adult-onset leukoencephalopathy with axonal spheroids and pigmented glia (ALSP) is a rare autosomal dominant neurodegenerative disorder that predominantly affects the cerebral white matter. It was coined as a comprehensive pathological term for the previously known terms pigmentary orthochromatic leukodystrophy (POLD) and hereditary diffuse leukoencephalopathy with spheroids (HDLS) [1]. In 2012, a mutation in the colony-stimulating factor-1 receptor (*CSF1R*) gene encoding CSF1R was identified in patients with HDLS [2, 3].

Later, Nicholson et al [1] also identified *CSF1R* mutations in two pathologically diagnosed POLD families, providing genetic evidence that POLD and HDLS can be categorized as the same disease entity as ALSP. ALSP is clinically characterized by two cardinal components: motor and neuropsychiatric symptoms [4]. The former symptoms include parkinsonian symptoms, upper motor neuron signs, bulbar signs, and ataxia; the latter symptoms include progressive cognitive decline, depression, apathy, anxiety, irritability, and other behavioral changes [5]. Typically, the clinical onset of symptoms occurs around the fourth decade of life, and the

survival time ranges from months to decades [6]. The key neuropathological characterizations are extensive white matter degeneration with a loss of myelin sheaths and axons, abundant neuroaxonal spheroids, and lipid-laden and pigmented macrophages [2, 7]. Before discovering the causative gene, only a few cases with ALSP were reported because a neuropathological examination was required to determine the diagnosis.

The main causes of ALSP were identified as *CSF1R* gene mutations [3, 8]; *CSF1R* encodes a cell surface receptor that plays important roles in development and innate immunity by regulating the development of macrophages in most tissues. CSF1R protein is predominantly expressed in microglial cells in the brain; therefore, microglial dysfunction caused by *CSF1R* mutations is believed to play a key role in the pathogenesis of ALSP [9]. Due to the rapid advances in molecular genetics, the discovery of causative genes facilitates the increasing recognition of ALSP. Nevertheless, patients with genetic diagnoses are still rare, and many patients may still be underdiagnosed. To date, different mutations of *CSF1R* have been identified, including small indels and missense, frameshift, splicing site, and regulator site mutations. The haplo-insufficiency of *CSF1R* is evident in patients with frameshift mutation [10]. Here, we report an ALSP family with a novel splicing site mutation (c.2319+1C>A) of the *CSF1R* gene, which resulted in a dominant-negative function of this gene.

Materials and methods

Research ethics

The study was approved by the Institutional Review Board of the Affiliated Hospital of Xuzhou Medical University, and informed consent was obtained from family members.

Clinical and neuroimaging characteristics of the patients

Clinical presentations and neuroimaging findings of the 3 patients were retrospectively evaluated. Genetic analyses were conducted under the approval of the ethics committee. The detailed clinical phenotypes were obtained according to the previous description [11, 12]. The information included sex, age of onset, age at exam, duration (if applicable), initial symptoms, brain imaging features, and the type of mutation. Magnetic resonance imaging (MRI) was conducted for routine diagnostic purposes using 1.5T or 3.0T systems. T1 weighted imaging (T1WI), T2 weighted imaging (T2WI), fluid attenuated inversion recovery (FLAIR), diffusion weighted imaging (DWI) and brain CT were available in all patients. Susceptibility weighted imaging (SWI) and magnetic resonance angiography (MRA) were available in one patient.

Mutation analyses of the NOTCH3 gene

Genomic DNA was isolated from peripheral leukocytes using a QIAamp DNA Blood Mini Kit. All exons and their flanking intronic sequences of the *NOTCH3 gene* were amplified by polymerase chain reaction (PCR) using 2×Hieff™ HotStart PCR Master Mix (With Dye). PCR primers were described previously [13]. PCR products were directly sequenced by the Sanger method. DNA sequences were analyzed by comparing them to the February 2009 human reference sequence (GRCh37/hg19) with the BLAT tool from the UCSC Genome Browser.

Whole-exome sequencing

Genomic DNA samples were purified from peripheral leukocytes. The genomic DNA samples were used for exome capture using the Roche NimbleGen human exon V2 capture chip (Roche, Pleasanton, CA, USA) according to the manufacturer's protocols, and sequencing data were obtained using the Roche NimbleGen human exon V2 capture chip on the Illumina HiSeq 2500 platform (Illumina Inc.). We created a single variant call format by merging the calls from each sequencing platform and filled in the reference sequences (nonvariable positions) using a jointly called variant call format from the two platforms. The putative mutation of the *CSF1R* gene was validated by PCR and Sanger sequencing in the available family members. The PCR primers used were as follows: forward primer: 5'-TGAGGAAGCTTCCGTTATCA-TC-3'; reverse primer: 5'-TCTCTCCTATGTAGG-GACAGTGG-3'.

Quantitative real-time PCR (q-PCR)

Total RNA was isolated from peripheral blood using RNA iso Plus (Takara Company, Japan). Purified RNA (~500 ng) was reverse transcribed
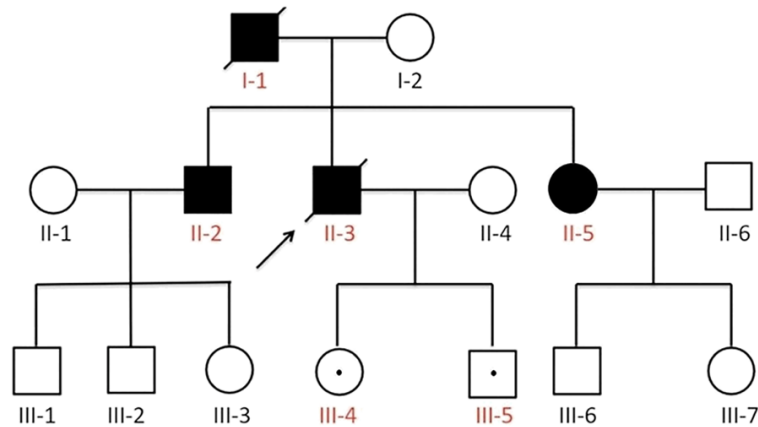
Figure 1. The pedigree of the family with ALSP is shown. There were three generations in this family. Affected patients are represented by filled black symbols. An arrowhead indicates the proband. Red symbols represent carriers of the c.2319+1C>A mutation. Open symbols indicate obligate gene carriers currently without symptoms. Diagonal lines through symbols denote deceased individuals.

using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, US). Primer sequences for q-PCR are shown in Supplementary Table 1. q-PCR was performed with SYBR Premix Ex Taq (Takara) in a CFX Connect™ Real-Time PCR Detection System. The relative levels of mRNA were normalized to β-actin mRNA expression for each sample using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

All data are presented as the mean ± SD. Student's t-test was conducted to identify differences between two groups. The difference was considered statistically significant at P<0.05. GraphPad Prism version 5.0 (GraphPad, San Diego, CA, USA) was applied for statistical analyses.

Results

Patient history and clinical features

The family pedigree of this Chinese ALSP family is shown in **Figure 1**. Analysis revealed an autosomal dominant hereditary pattern in this family. Three patients carrying CSF1R variants were retrospectively evaluated. We examined the clinical and neuroradiological features of the family. No significant risk factors for cerebrovascular disease were found in any of the subjects. Each patient presented with different initial symptoms and was misdiagnosed. The mean age of symptom onset in the family was

54.7 years. II-1 and II-3 died during the study; II-5 gradually showed a mild decline in cognitive function.

Patient 1 (II-3)

The male patient (II-3) was the proband. At the age of 41 years, his main complaints were gait disturbance and rigidity, and his Babinski sign was positive. He was diagnosed with cervical spondylotic myelopathy and underwent surgical treatment at a local hospital. However, the symptoms did not improve but progressed. After half a year, he started having difficulty speaking and was hospitalized. The patient denied any disease history before. In his family history, his father had memory impairment. There was no history of medication or substance abuse. In his physical examination, he displayed influent speech, slight left facial nerve palsy, high spasticity with dyskinetic movements of limbs, ataxic gait, brisk tendon reflexes at both knees and ankles, and bilateral positive Babinski signs. His Mini-Mental State Examination (MMSE) score was 20/30. The results of blood routine analysis, thyroid function tests, metabolic investigations, and vasculitis screening were all normal. The electroneuromyography and the electroencephalogram did not reveal any positive findings. MRI showed confluent hyperintensities on T2W and FLAIR images and slightly hypointensity on T1W images symmetrically in the periventricular white matter and indicated a thin corpus callosum (**Figure 2A-C**). MRI also showed ventricular abnormalities, including cavum septum pellucidum and cavum vergae (**Figure 2A**, **2B**), and strikingly restricted diffusion (**Figure 2D**). No obvious abnormalities were found in the cerebral arteries in MRA. No calcification was shown on brain CT. His condition worsened and he died of pneumonia at 44 years of age. The duration of the disease was only 3 years.

Patient 2 (II-2)

This patient (II-2) was the older brother of the proband (patient 1); he was 52 years old and a
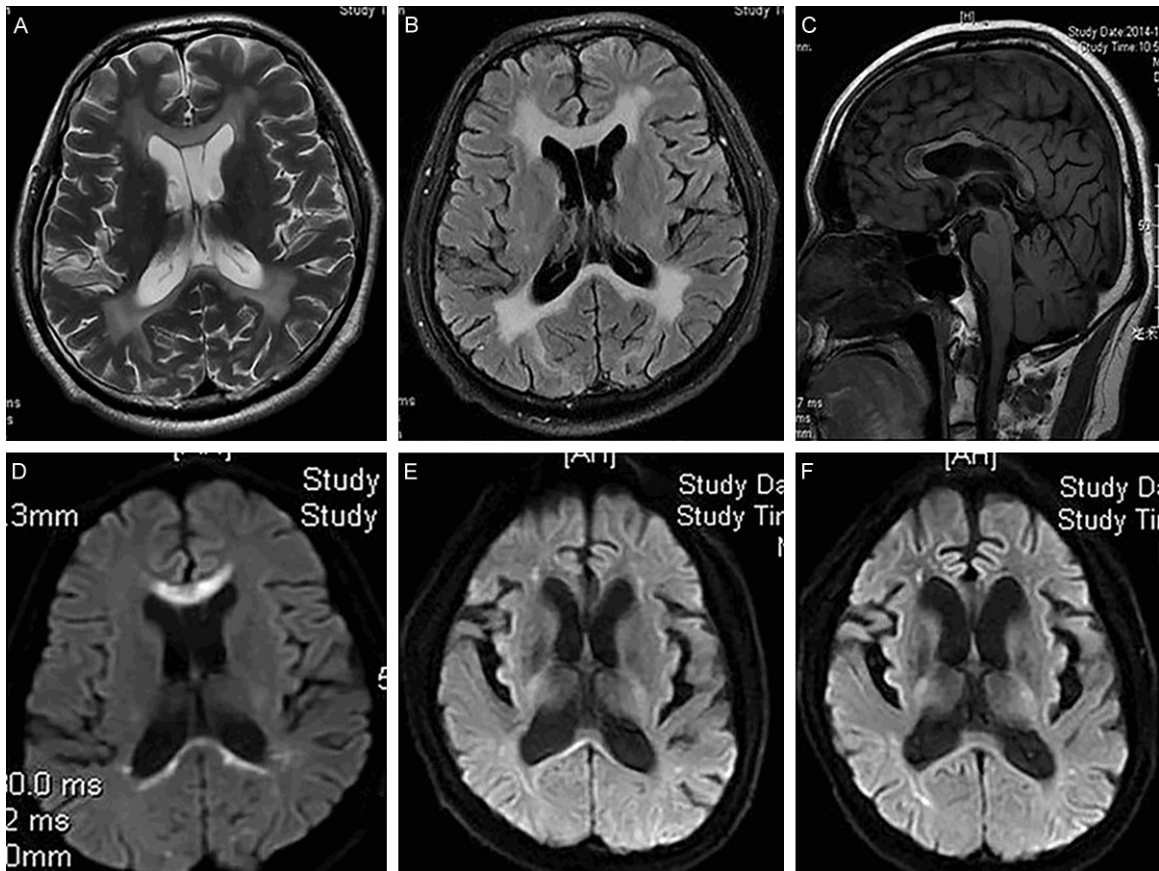
Figure 2. The imaging findings in the patients with ALSP on MRI are shown. MRI findings in the proband (II-3) are shown in (A-D). T2 sequencing showed hyperintensity in the deep and periventricular white matter, cavum septum pellucidum, and cavum vergae (A). FLAIR imaging sequencing showed typical leukoaraiosis (B). Sagittal T1WI sequencing showed the thin corpus callosum (C). DWI sequencing showed restricted diffusion in the splenium of the corpus callosum (D). MRI findings in patient 2 (II-2) are shown in (E, F). DWI sequencing demonstrated restricted diffusion in the punctate areas (E), and these lesions were persistent for more than 10 months (F).

truck driver working normally when his young brother was admitted to the hospital in 2014. The patient saw the doctor in 2016 because of his emotional abnormalities; he was very irritable and had gradual memory loss. Physical examination showed cognitive decline, anxiety, and irritability but no obvious motor symptoms. At the age of 55, he had muscle weakness in the left upper limb, spasm in the left limbs, brisk tendon reflexes at both knees and ankles, and bilateral positive Babinski signs. His MMSE score was 16/30. All blood tests were negative. His MRI findings were very similar to those of his younger brother (II-3) and revealed severe atrophy of the cerebral white matter and the corpus callosum as well as enlarged lateral ventricles. Unlike the ischemic lesions, diffusion-restricted lesions of this patient were persistent for several months, presumptively

reflecting intramyelinic edema [14] (**Figure 2E, 2F**). Multiple low-signal lesions were not seen on SWI, which was different from the imaging of CADASIL. Brain CT revealed no calcification. Recent follow-up showed the deterioration of neuropsychiatric symptoms and motor disorders, including bradykinesia, postural instability, pyramidal signs and ataxia.

Patient 3 (I-1)

This patient (I-1) was the father of the proband (II-3). In 2011, when he was 70 years old, he was often found to lose his way because of memory decline. In subsequent years, the patient gradually showed abnormal mood, ataxic gait, and poor control of urine and stool. Then, a suspected diagnosis of Alzheimer's disease was made. In 2016, he showed severe

Table 1. Summary of the clinical and neuroimaging characteristics

Patient no.	1	2	3
Sex	M	M	M
Age at onset (y)	40	54	70
Age at exam (y)	41	54	75
Duration (y)	1		6
Initial symptoms	Gait disturbance	Emotional abnormality	Decline in memory
Initial diagnosis	Cervical spondylotic myelopathy	Binswanger disease	Alzheimer's disease
Clinical features			
Psychiatric symptoms	-	+	+
Cognitive impairment	+	+	+
Parkinsonism	+	+	+
Pyramidal signs	+	+	+
Additional features	-	-	Bladder dysfunction
Brain imaging features			
Bilateral white matter lesions	+	+	+
Thinning of the corpus callosum	+	+	+
Diffusion-restricted lesions	+	+	-
Microbleeds on SWI	n.a.	+	n.a.
Dilation of the lateral ventricles	+	+	+
Calcifications in the white matter	-	-	-
Genetic testing	c.2319+1C>A	c.2319+1C>A	c.2319+1C>A

cognitive decline, apathy, irritability and postural instability. In 2017, he was bedridden because of limb rigidity and had also dysphagia and convulsions. He died of pneumonia at the age of 76. The duration of the disease was 6 years. His CT and MRI findings were very similar to those of his sons. Additionally, brain CT revealed no calcification. Detailed information for the three patients is summarized in **Table 1**.

Genetic analysis of this family

Initially, patients in the family were misdiagnosed with CADASIL because of MRI imaging and a dominant hereditary pattern within the family history. Therefore, we examined the *NOTCH3* gene and found no pathogenic mutation, indicating that the diagnosis of CADASIL was incorrect. Further, we performed whole-exome sequencing in the proband (II-3) and his father (I-1). The results showed a splicing mutation, c.2319+1C>A, of the *CSF1R* gene, supporting the rare autosomal dominant disease ALSP. Combined with the sequencing results of more than 60,000 total exon sequences in the Exome Aggregation Consortium database, this mutation was not found. The mutation was also not found in the UCSC Genome database, 1000

Genomes, the Human Gene Mutation Database, or other professional databases. In addition, this mutation was validated in the proband's brother (II-2) and in II-5, III-4, and III-5, as shown in **Figure 3** (except for II-2 and II-5, the other individuals had no symptoms at last visit), but not in other family members or in 100 healthy controls. To demonstrate whether this mutation could cause nonsense-mediated RNA decay (NMD), we examined the expression of CSF1R mRNA in the blood of the available family members by real-time quantitative PCR. For patient II-2, the level of CSF1R mRNA was remarkably lower than that of the healthy control subjects as determined by multiple primer pairs from different exons of the *CSF1R* gene (**Figure 4A**). Similar lower levels (~15%) of CSF1R mRNA were also found in the other three individuals (II-5, III-4 and III-5) who carried the mutations compared with the six healthy control subjects (**Figure 4B**, P<0.05).

Discussion

Almost all the previously reported mutations in ALSP were found within exons 12-22, including the coding sequence of the tyrosine kinase domain (TKD) [3, 10, 11]. In this study, we iden-
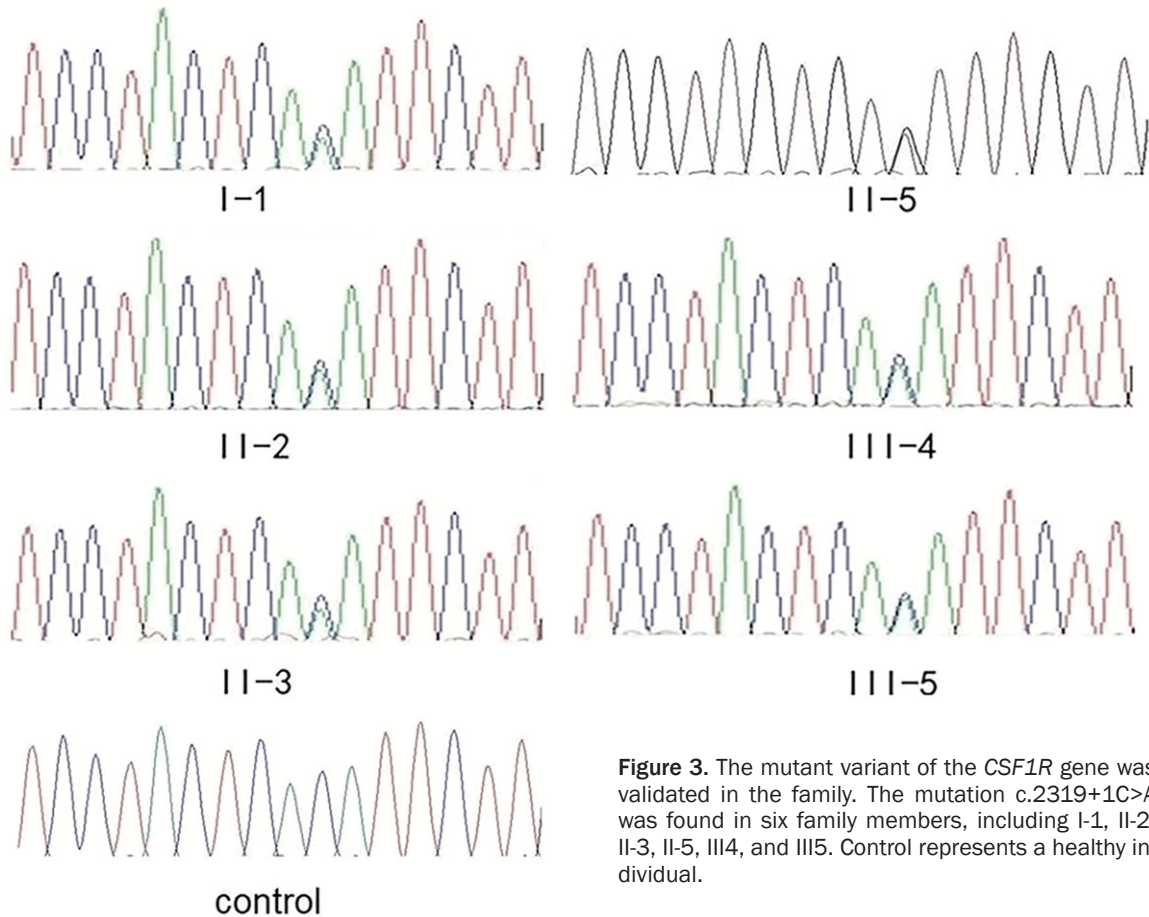
Figure 3. The mutant variant of the *CSF1R* gene was validated in the family. The mutation c.2319+1C>A was found in six family members, including I-1, II-2, II-3, II-5, III4, and III5. Control represents a healthy individual.
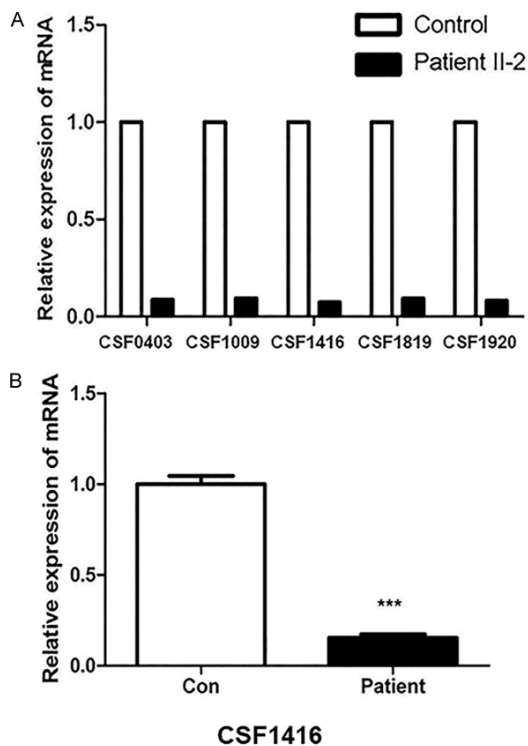

Figure 4. mRNA expression of the *CSF1R* gene in peripheral blood. The *CSF1R* gene mRNA expression was measured by quantitative PCR with different primers for the different segmentation of the *CSF1R* gene mRNA in patient 2 (II-2, A). The *CSF1R* gene mRNA expression was measured in six control subjects and four individuals carrying the novel splicing mutation using huCSF1R1416 (B).

tified a novel splicing mutation in intron 16 of the *CSF1R* gene in patients with ALSP who fulfilled the diagnostic criteria [15]. The novel splicing mutation c.2319+1C>A was located at the splice site within the TKD, consistent with previous reports. The expression analysis of CSF1R mRNA confirmed the pathogenesis of this splicing mutation of the *CSF1R* gene. Since the TKD is an important intracellular component of CSF1R, ligands such as CSF1 or interleukin-34 bind to CSF1R and subsequently activate the TKD through autophosphorylation [16]. The activation of the TKD is necessary to initiate signal transduction, which contributes to the development, maintenance

and activation of microglia [17]. A loss of function of the CSF1 receptor by mutations of the TKD results in the decreased activation of CSF1R and aberrant microglia density or the early loss of microglia, followed by the phenotypes of ALSP [18].

The majority of ALSP patients presented with variable phenotypes, including cognitive impairment, neuropsychiatric disturbance, and pyramidal and extrapyramidal symptoms. Due to the heterogeneity of clinical phenotypes, many patients with ALSP are often clinically misdiagnosed with Alzheimer's disease, frontotemporal lobar degeneration (FTLD), corticobasal degeneration, atypical parkinsonism, CADASIL or primary-progressive multiple sclerosis [3, 11, 19, 20]. In this study, the proband was misdiagnosed with cervical spondylotic myelopathy and CADASIL before the genetic testing that pointed to the final diagnosis of ALSP. In this family, initial symptoms included gait disorder (motor dysfunction), psychiatric symptoms or cognitive impairment. Three patients had different symptoms. The age of onset varied greatly, ranging from 40 to 70 years. None of these cases showed previously reported symptoms such as stroke-like episodes, sensory disturbance, dizziness, fatigue, epilepsy or peripheral neuropathy. Furthermore, III-4 and III-5, who carry the mutations, are asymptomatic at present, perhaps because they are still in their teens. We will give more attention to their further clinical follow-up.

To date, relatively distinctive imaging findings of ALSP have been identified, including extensive white matter involvement, persistent small diffusion-restricted lesions, a thin corpus callosum, and deep punctate calcifications [15, 21]. Some studies have demonstrated that small calcifications with a stepping-stone distribution are characteristic of the disease [22]. Notably, these calcifications are not normally distributed within the basal ganglia, thalamus, and cerebellum, which may help to differentiate ALSP from other genetic or metabolic diseases [23]. However, no calcification was found in the patients of this family, suggesting that brain calcification may be associated with certain genotypes of ALSP or that thinner-slice CT scanning is needed to determine the calcification.

Considering that pathological assessment is not feasible and imaging findings are not spe-

cific for the diagnosis of ALSP in many cases, clinicians are apt to rely on molecular genetics. Therefore, a mutational analysis of the *CSF1R* gene should be considered for those patients who are clinically suspected to have leukoencephalopathy. In this study, we detected a novel c.2319+1C>A mutation in the splice site of the *CSF1R* gene by whole-exome sequencing that was validated in other family members. CSF1R mRNA was significantly decreased (~15%) in the peripheral blood samples of affected patients, which was much lower than the expected 50%. Surprisingly, the CSF1R mRNA level was 15% lower than that of the healthy controls, which is not as much as the ~50% that we expected in the family carriers, which indicated that the mutation not only caused the NMD with this mutant allele but also resulted in the degradation of the wild type allele, confirming the pathological nature of this splice-site mutation of *CSF1R*. This result indicated that the novel splicing mutation c.2319+1C>A of the *CSF1R* gene is a dominant-negative mutation. This may contribute to the specific phenotypes of this family, such as no brain calcification on CT imaging.

In summary, c.2319+1C>A splice-site mutation was identified as a cause of ALSP in this family. Our findings have extended the genetic spectrum of ALSP with *CSF1R* mutations and broadened the clinical spectrum of this disease.

Disclosure of conflict of interest

None.

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Supplementary Table 1. Primers for real-time fluorescence quantitative PCR

Primer name	Sequence (5' to 3')
huCSF1R0304F	ACC AGC TCT GCA GGC ACC AG
huCSF1R0304R	AGC CAG GAC TAT CAA TGC AGT GC
huCSF1R1009F	GCC ATG GAC ATG CAG G
huCSF1R1009R	GAG CCT GCT GAC TGT TGA GAC C
huCSF1R1416F	CCA CAT GAC CAT TGG TCA ACA GC
huCSF1R1416R	CAC CTA TGT GGA GAT GAG GCC TG
huCSF1R1819F	GCC ATT TGG TAT CCA TCC TTC AC
huCSF1R1819R	ACG TCT GGT CCT ATG GCA TCC TC
huCSF1R1920F	GGA GCA GAT CTG GAA GGT G
huCSF1R1920R	GTG AAG GAT GGA TAC CAA ATG GC